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I, KIM MARSHALL, MANAGER EXAMINATION SUPPORT AND SALES, hereby certify that the annexed is a true copy of the Provisional specification in connection with Application No. PP 4665 for a patent by THE UNIVERSITY OF NEWCASTLE RESEARCH ASSOCIATES LIMITED filed on 14 July 1998.

WITNESS my hand this Thirtieth day of July 1999

KIM MARSHALL
MANAGER EXAMINATION SUPPORT AND SALES

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AUSTRALIA

Patents Act 1990

PROVISIONAL SPECIFICATION

Applicant(s):

THE UNIVERSITY OF NEWCASTLE RESEARCH ASSOCIATES LIMITED

A.C.N. 000 710 074

Invention Title:

Product and Process

The invention is described in the following statement:

Compounds and Methods of Treatment

Technical Field

This invention relates to compounds useful in the treatment of certain forms of cancer; processes for producing these compounds; methods of treatment using these compounds *per se;* methods of treatment using these compounds which methods also increase the sensitivity of cancer cells to other treatments; methods of screening these compounds for anti-cancer activity; and methods of screening these compounds for anti-cancer activity and/or ability to sensitise cancer cells to other methods of treatment. More particularly, the compounds are specific inhibitors of protein phosphatases 1 and 2A.

Background Art

Protein Phosphatase inhibitors and the Abrogation of the G_2 Checkpoint

The regulation of protein phosphatases is integral to the control of many cell processes, including cell growth, transformation, tumour suppression, gene transcription, apoptosis, cellular signal transduction, as neurotransmission, muscle contraction, glycogen synthesis, and T-cell activation. The role of protein phosphatases in many of these processes is often mediated via alterations in the cell cycle. Cell cycle progression is tightly regulated to ensure the integrity of the genome. During cell division it is imperative that each stage of the cell cycle be completed before entry into the next, and this is achieved through a series of checkpoints.

Following DNA damage induced by chemotherapy or radiation treatment these checkpoints are responsible for halting cell cycle progression in G_1, S and/or G_2 phases (O'Connor, 1996). The cell undergoes a cell cycle arrest so that the damaged DNA can be repaired before entry into S phase or mitosis. The phase at which the cell cycle is halted will depend upon the type of DNA damaging agent used and the point during the cell cycle that the damage was incurred (O'Connor, 1997).

Serine/threonine phosphatases, which are responsible for protein dephosphorylation, comprise a unique class of enzymes consisting of four primary subclasses based on their differences in substrate specificity and environmental requirements. Of the serine/threonine phosphatases, protein phosphatases 1 and 2A (PP1 and PP2A, respectively) share sequence identity between both enzyme subunits (50% for residues 23-292; 43% overall), are present in all eukarytoic cells and are together responsible for 90% of all cellular dephosphorylation. Knowledge of structure and subsequent correlation of binding function for both PP1 and PP2A would therefore provide a vital link toward understanding the biochemical role of these enzymes. A goal of the medicinal chemist is the development of potent and selective inhibitors of these protein phosphatases.

The natural toxins, okadaic acid, calyculin A, microcystin-LR and tautomycin are representative of a structurally diverse group of compounds that are all

potent protein phosphatase 1 (PP1) and 2A (PP2A) inhibitors. Okadaic acid is more specific for PP2A (IC_{50} 1nM) than PP1 (IC_{50} 60nM), while calyculin is slightly more specific for PP1 (IC_{50} 0.5-1.0nM) than PP2A (IC_{50} 2nM). All of these phosphatase inhibitors are known to abrogate the G_2 checkpoint of the cell cycle and induce cellular mitoses (Yamashita et al., 1990). Abrogation of the G_2 checkpoint means that the cell does not have the capacity to detect DNA damage or malformation of the genome prior to entry into mitosis. Therefore, cells which have a deficient G2 checkpoint are unstable, and incapable of detecting DNA damage, initiating G2 arrest, or undergoing DNA repair. Such cells enter the mitotic stage of the cell cycle prematurely with malformed spindles. The abrogation of the G_2 checkpoint in the cell cycle by okadaic acid is mediated via the activation of cdc2/H1 kinase, the major mitotic inducer, and results in a premature mitotic state (Yamashita et al., 1990). Although okadaic acid is known as a tumour promoter, in some cell types, it has been shown to revert the phenotype of oncogene-transformed cells to that of normal cells, and to inhibit neoplastic transformation of fibroblasts (Schonthal, 1991).

Furthermore, okadaic acid has been shown to selectively enhance the cytotoxicity of vinblastine and the formation of apoptotic cells, in HL60 cells which are p53 nul (Kawamura, 1996). Interestingly, calyculin enhances irradiation killing in fibroblast cells at doses that are non toxic when given as a single treatment.

(Nakamura and Antoku, 1994).

The okadaic acids class of compounds, with the exceptions of okadaic acid, cantharidin (Honaken) and thyrisferyl 23-acetate (Matszawa et. al) (being PP2A selective) they exhibit poor selectivity. Furthermore, the concentration of PP1 and PP2A inside cells is such that high concentrations of these inhibitors are required to generate a response *in vivo* resulting in the loss of effectiveness of any *in vitro* selectivity (Wang).

Cantharidin (exo, exo-2,3-dimethyl-7-oxobicyclo[2.2.1]heptane-2,3-dicarboxylic acid anhydride), is a major component of the Chinese blister beetles: *Mylabris phalerata* or *M. cichorii*)(Yang; Cavill et. al). The dried body of these beetles has been used by the Chinese as a natural remedy for the past 2000 years. Although Western medicine decreed cantharidin to be too toxic in the early 1900's (Goldfarb et. al) its purported aphrodisiac qualities (the active ingredient of "Spanish Fly"), and its widespread occurrence in cattle feed still results in numerous human and livestock poisonings (Schmitz).

Li and Casida, and previous work in this laboratory (McCluskey et. al) (and more recently Pombo-Villar, Sodeoka) has assisted in the delineation of certain features crucial for inhibition of PP2A by cantharidin analogues (Figure 1). However the corresponding picture for PP1 is not so clear, the majority of data refers to possible interactions with the known crystal structures,

and in some cases the inhibition values for PP1 are not reported.

Involvement of Tumour Suppressor Gene p53

The most commonly mutated gene in human cancers is the tumour suppressor gene p53, which is abnormally expressed in more than 50% of tumours. The development of chemotherapeutic agents which selectively target cancer cells with mutant p53 is certainly desirable, for two main reasons. Firstly, cells that have an abnormal p53 status are inherently resistant to conventional chemotherapy and produce the more common, and more aggressive tumours such as colon carcinoma and non small cell lung cancer. Secondly, a chemotherapy regime that targeted only those cells with a mutant p53 phenotype would potentially produce fewer side effects since only the cancer cells would be killed and not the p53 proficient normal healthy cells.

Disclosure of the Invention

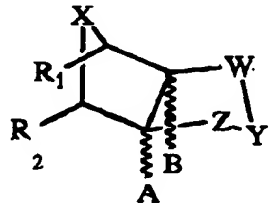
In relation to the discussion above and on the basis of previous efforts, we believed that the replacement of the ether O atom of the anhydride with N or S (as N-H and N-R, where R = alkyl or aryl) would allow us to probe the H-bonding requirements of this region of cantharidin analogues. Previous studies in our laboratory had shown limited tolerance for modification of the 7-oxa position. An ability to modify these heteroatoms is crucial to the development of selective inhibitors based on this simple skeleton.

There is not, at present, an inhibitor with either absolute specificity or high enough selectivity which renders the inhibitor effectively specific in vivo.

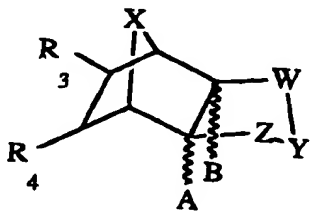
It has surprisingly been found that anhydride modified cantharidin analogues, which are the subject of this invention, are potent, selective, oxidatively stable, and cell permeable inhibitors of protein phosphatases 1 and 2A.

Therefore, according to the first aspect of this invention there are provided cell permeable inhibitors of protein phosphatases 1 and 2A, said inhibitors being anhydride modified cantharidin analogues, and possessing the properties of being potent, selective and oxidatively stable.

According to a particular embodiment of the first aspect of this invention there are provided compounds of the formula:



wherein R_1 and R_2 are H, aryl or alkyl; Y is O, S, SR, NH, NR, CH_2OH, CH_2OR; R is alkyl or aryl; A and B are H or CH_3; W and Z are CHOH or C=0 and R_1 and R_2 can cyclise to form a ring as follows:



wherein R_3 and R_4 are H, aryl or alkyl.

The aryl group may suitably be phenyl or naphthyl for example, and may be attached via a carbon spacer of between 6 and 10 carbon atoms. The alkyl group may suitably be C_1-C_{10}.

According to the second aspect of this invention there is provided a process for producing anhydride modified cantharidin analogues, said process including the steps of:

dissolving a diene in a suitable solvent and adding to the resultant solution an ene.

According to a particular embodiment of the second aspect of this invention, the reaction conditions are dependent on the aromaticity of the starting diene. These reaction conditions will be set out below.

According to the third aspect of this invention there is provided a method of treating a cancer which method comprises administering to a patient in need of such treatment, an effective amount of an anhydride modified cantharidin analogue of the first aspect of this invention, together with a pharmaceutically acceptable carrier, diluent and/or excipient.

According to the fourth aspect of this invention there is provided a method of sensitising cancer cells to at least one other method of treating cancer, which method of sensitising comprises administering to a patient in need of such treatment, an effective amount of an anhydride modified cantharidin analogue of the first

aspect of this invention, together with a pharmaceutically acceptable carrier, diluent and/or excipient, and utilising a second method of treatment.

According to the fifth aspect of this invention there is provided a method of screening a compound for anti-cancer activity.

According to the sixth aspect of this invention there is provided a method of screening compounds for use in the fourth aspect of this invention, said method comprising screening for anti-cancer activity; screening for ability to abrogate the G_2 checkpoint of the cancer cell cycle; and screening for the ability of said compounds for sensitised cancer cells to cisplatin and irradiation.

The process for producing the compounds of this invention generally may be stated thus:

As mentioned, the reaction conditions depend on the aromaticity of the starting diene. This is illustrated by a description of examples of the methods wherein the starting materials are furan. (Method 1 below); thiophene (method 2 below); and pyrrole (method 3 below).

Method 1: Furan as the starting diene

A solution of furan (5 equivalents) is dissolved in a suitable solvent (about 5 times the volume of furan, the solvent can be for example, ether (for room temperature reactions); or benzene or xylene (the latter two for reactions at 80 and 130°C respectively). To this solution is added one equivalent of the ene. The reaction is then heated (or stirred at room temperature), typically for 24

hours (2 days in the case of the room temperature reaction). Upon cooling (or standing at room temperature) a precipitate forms and is collected by vacuum filtration. The adduct is then purified by recrystalisation from for example, chloroform or ethanol. In the case of the furan + maleic anhydride compound care is exercised to minimise heating as this causes a reto-Diels-Alder reaction yielding only the starting materials.

Method 2: Thiophene as the starting diene

Thiophene (1.016g, 0.012 mol) and maleic anydride (0.558.0.006 mol) are mixed at room temperature in 2.5 mL of distilled dichloromethane. This mixture is then placed inside a high pressure reactor. They are compressed to a pressure of 17kbar at 40°C for a period of 71 hours, after which the pressure is released and the product purified by chromatography.

Method 3: Pyrrole as the starting diene

To $[Os(NH_3)_5OsO_2CF_3)](CF_3SO_3)_2$, (0.3511 g, 0.4 mmol) and activated magnesium (0.1511 g), pyrrole (0.45 mL, 0.6 mmol), DME (1 mL) and DMAc (0.3 mL) are added in that order. The mixture is stirred for 1 hour, the temperature gradually rising to 40°C and then dropping. The brown slurry is filtered through a thin pad of celite, and the cake washed with DME in small portions (4 x 2 mL). The filtrate is added to dichloromethane (15 mL). Vigorous stirring results in the formation of yellow coloured precipitate which is collected by vacuum filtration, followed by an ether wash (2 x 2.5 mL). The product is

dried under a stream of nitrogen yielding a yellow-tan solid (0.343g, 84%). To this pyrrole complex is added maleimide (0.05g, 0.515 mmol) (or any other "ene", eg maleic anhydride, dimethyl maleate, etc) in acetonitrile. The mixture is allowed to stir at room temperature for 60 min. after which the solvent is removed by vacuum, yielding the exo isomer (0.359g, 64%). The crude material is purified by ion-exchange column (Sephadex-CM C-25, 2 x 10 cm), using NaCl as the mobile phase. The complexes are precipitated by the addition of a saturated sodium tetraphenylborate solution.

The types of cancer which are amenable to treatment by these compounds include those types of cancer which are inherently resistant to conventional chemotherapy. Typically, these types of cancer are represented by the more common and more aggressive tumour types such as, but not limited to, colon cancer and non small-cell lung cancer.

The compounds of this invention are suitably administered intravenously, although other modes of administration are possible. Pharmaceutically acceptable diluents, adjuvants, carriers and/or excipients may be used in conjunction with the compounds of this invention.

The compounds of this invention may also sensitise cancer cells to other methods of treatment. For example, typically these methods include irradiation and treatment with platinum anti-cancer agents, for example cisplatin.

In addition, sensitisation may also be brought about

by, for example the use of the plant alkaloids vinblastine and vincristine, both of which interfere with tubulin and the formation of the mitotic spindle.

In particular, the compounds of this invention sensitise those cells with deficient p53 activity.

With regard to the fifth aspect, when screening for anti-cancer activity, various cancer cell lines are chosen. These are typically both haematopoietic and solid tumour cell lines with varying p53 status and include: L1210 (murine leukaemia, p53 wildtype), HL60 (human leukaemia, p53 nul), A2780 (human ovarian carcinoma, p53 wildtype), ADDP (cisplatin resistant A2780 cells, p53 mutant), SW480 (human colon carcinoma, p53 mutant) and WiDr (human colon carcinoma, p53 mutant).

With regard to the sixth aspect, in addition to the methods for screening for anti-cancer activity, the following procedures are suitably used in the remainder of the screening process. For example, when screening for the ability to abrogate the G_2 checkpoint of the cancer cell cycle, the following methods are suitably used:

Cell cycle method

The cells are fixed in 70% ethanol and stored at -20°C until analysis is performed (1-2 weeks). After fixing, the cells are pelleted and incubated in PBS containing propidium iodide (40mg/ml) and RNase A (200 mg/ml) for at least 30 min at room temperature. The samples (2 x 10^4 events) are analysed using a Becton Dickson FACScan, fluorescence is collected in fluorescence

detector 2 (FL2), filter 575/30 nm band pass. Cell cycle distribution is assessed using Cell Quest software (Becton Dickson).

Those protein phosphatase inhibitors which show abrogation of the G_2 checkpoint are then be exploited in combination studies with either radiation exposure or cisplatin incubation. The MTT (3-[4,5-dimethylthiazol-2-yl]2,5-diphenyl-tetrazolium bromide) assay is used to determine whether a synergistic, antagonistic or additive effect is induced. The Median Effect method is adopted to mathematically determine the optimal combination index of the treatments chosen (Chou and Talalay, 1984). This method has been extensively used to investigate the cytotoxicity of various drug combinations including cisplatin and D1694 (Ackland et al 1996;1998). A combination index value less than 1 indicates synergism, a value equal to 1 indicates additivity and a value greater than one indicates antagonism.

Cytotoxicity assay

When screening for the ability to sensitise cancer cells to cisplatin and irradiation, the following methods are suitably used:

Cells in a subconfluent phase are transferred to 96-well microtitre plates. L1210 cells are plated at a density of 1000 cells/well in 100µl medium, while all other cell lines are plated at a density of 2000-25000 cells/well. The cells are left for 24h prior to treatment to ensure exponential growth has been achieved, 24h after

plating (day 0), 100µl of phosphatase inhibitor is added to each well, control wells received 100µl of medium only. Drug exposure time is 72h (day 3). The effect of phosphatase inhibition is tested in triplicate over a concentration range of 1×10^{-3}M - 1×10^{-8}M. Growth inhibitory effects are evaluated using the MMT assay and absorbance read at 570 nm. The IC_{50} is the drug concentration at which cell growth is 50% inhibited based on the difference of optical density on day 0 and day 3 of drug exposure. Cytotoxicity is evaluated using a spectrophotometric assay which determines the percentage of cell growth following exposure of the cells to various concentrations of the phosphatase inhibitors for a period of 72 hours. The subsequent dose response curve is used to calculate C_{60} values (the drug concentration at which cell growth is 50% inhibited).

Most drug discovery has focused on the development of new single agents. However, in light of the success of combination chemotherapy it is increasingly apparent that successful anticancer treatment of the future will be based upon the discovery of agents which are synergistic in their action. In view of this, the cytotoxicity of phosphatase inhibitors in combination with either radiation or cisplatin is examined. As indicated above, calyculin which by itself is not cytotoxic, enhances irradiation induced cell death. Similarly abrogation of the G_2 checkpoint by either, caffeine or UCN-01, also enhances the cytotoxicity of γ irradiation in cells with

mutant p53 (CA46 and HT-29 cells) (Powell et al., 1995; Russell et al., 1995; Wang et al., 1996). DNA damage induced by irradiation causes both a G_1 and G_2 cell cycle arrest. In p53 mutant cells, the G_1 checkpoint is absent. However, following irradiation the cells will still arrest in the G_2 phase, and potentially repair the damage. P53 mutant cells are generally more resistant to conventional chemotherapy and produce more aggressive tumours. Therefore, in p53 deficient cells, DNA damage that is not detected by the G_1 checkpoint will be picked up by the G_2 checkpoint. If the cells are deficient in both of these checkpoints then it is believed that the cells will be unable to initiate repair mechanisms and will be more unstable and increasingly susceptible to cell death induced by DNA damage.

Cisplatin is another commonly used anticancer treatment which binds to DNA and produces DNA crosslinks and strand breaks. Cisplatin is particularly useful in the treatment of testicular carcinoma, small cell carcinoma of the lung, bladder cancer, and ovarian cancer. Repair of cisplatin induced DNA damage is mediated via nucleotide excision repair which is coordinated by p53 activation of Gadd45 (Smith et al., 1994). In this context, it has been suggested that cells that are p53 mutant are more sensitive to cisplatin treatment (Hawkins et al., 1996). A number of researchers have investigated this proposal in p53 mutant cell lines and in p53 mutant tumours, with mixed results. While it is apparent that cisplatin is

more cytotoxic in cells lines that are deficient in p53 (induced via papillomavirus) compared to the p53 proficient cells (Hawkins et al., 1996), it is harder to test this hypothesis in tumours and in cisplatin-resistant cells as they may have several undefined mutations in their genome which would confound such studies (Herod et al., 1996). Nevertheless, the G_2 abrogator UCN-01 (7-hydroxystaurosporine, a protein kinase inhibitor) has been shown to markedly enhanced the cell-killing activity of cisplatin in MCF-7 cells defective for p53 function (Wang et al., 1996).

The development of chemotherapeutic agents which selectively target p53 mutant cells is desirable since 50% of tumours have either a mutated or deleted p53 gene. Many of these p53 deficient cells and tumours are inherently resistant to conventional chemotherapy and represent the common more aggressive tumour types such as *colon cancer, and non-small cell lung cancer*.

Brief Description of the Figures

Figure 1 is a schematic representation of the structure activity data generated for inhibition by PP2A by cantharidin analogues;

Figure 2 is a diagram of asynchronous L1210 cells and following γ-irradiation; and

Figure 3 sets out the results of serial dilutions of the compounds of this invention required to kill 50% of the cells specified in this figure.

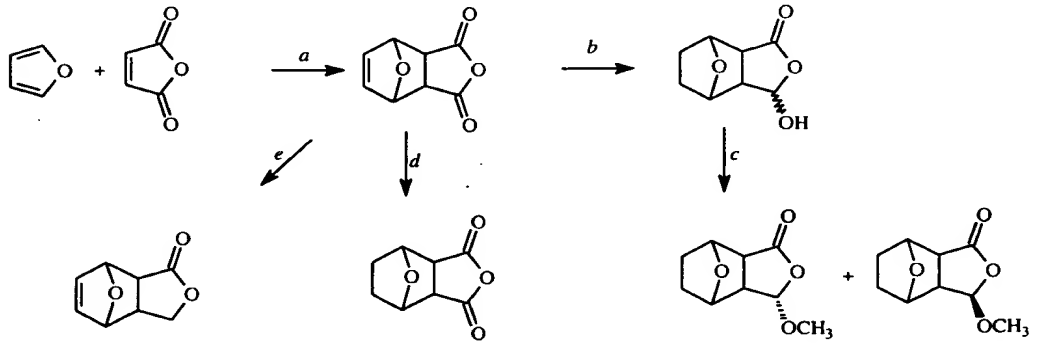
Best and Other Modes for Carrying Out the Invention

The invention is illustrated by way of the following examples which are not to be construed as limiting on the scope thereof.

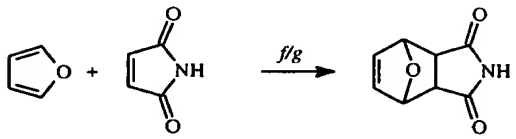
Example 1

Chemistry

Anhydride modified cantharidin analogues were synthesised by a variety of modified literature procedures, as set out in schemes 1 and 2. These modifications are embodied in the three methods, which depend on the aromaticity of the starting dienes, set out above. The dimethyl ester (**3**), which was prepared by the application of high pressure, 17kbar, 40°C 61 hours, as shown in scheme 3.

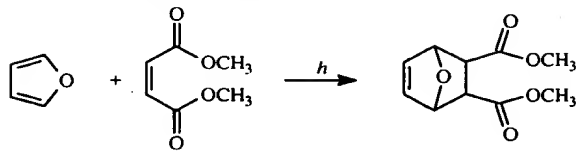


Scheme 1. *a*. Furan:maleic anhydride (5:1), diethylether, 2d, RT, 96%; *b*. H_2 / 10% Pd-C/ EtOH; *c*. *p*-TosOH, MeOH, chromatography; *d*. H_2 / 10% Pd-C/ Acetone; *e*. $NaBH_4$, then HCl.



Scheme 2. Reagents and Conditions: *f*. Furan:maleimide (5:1), diethyl ether, 7d, in dark, 75%, exo product; *g*.

Furan:Maleimide (5:1), diethylether, sealed tube 12h, 90ºC, 66%, endo product.



Scheme 3. Reagents and Conditions: *h*.

Furan:dimethylmaleate (2:1), CH_2Cl_2, 17 Kbar, 40ºC, 61 h, 56%.

Example 2

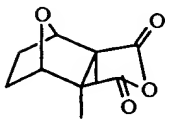
Development of potent, selective, oxidatively stable, and cell permeable inhibitors of protein phosphatases 1 and 2A.

Crude natural product extracts have yielded isopalinurin and a series of cantharidin analogues have been synthesised. In this context, the present inventors have developed the simple cantharidin analogue which is PP1 selective (IC_{50} = 50mM, with 0% inhibition of PP2A at concentrations ≥1000mM) representing the first small molecule to exhibit selectivity for PP1. Results have indicated that a series of simple synthetic modification of the cantharidin skeleton also allows the synthesis of a PP2A selective compound (see Figure 1 below).

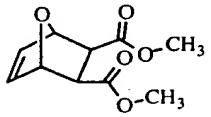
The present inventors have previously demonstrated that a facile ring opening of an anhydride is crucial to inhibition of PP2A, this is not possible with **c** (previous studies with the 7-O, and this analogue indicated considerable hydrolytic stability of the maleimide link). It is also interesting to note that endothal thioanhydride is three fold more potent than cantharidin, with the S

atom being an important factor. It is thus envisaged that the 7-S group presents itself to the active sites metals and the N-H of the maleimide occupies the hydrogen bond cavity normally reserved for the 7-O substituent of cantharidin.

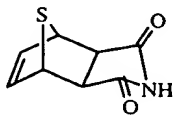
Structure of cantharidin and selective analogues



(a)



(b)



(c)

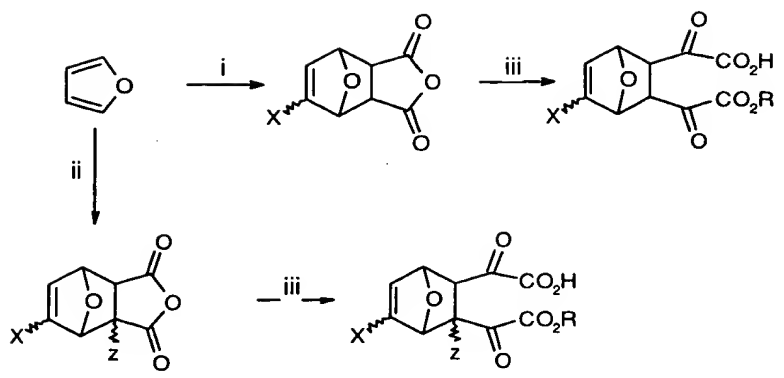
(a) Shows structure of cantharidin;

(b) Shows PP1 selective analogue; and

(c) shows PP2A selective analogue. Note that these are preliminary results (conducted in triplicate three time, but in the case of pannel (c) IC_{50} ~ 25mM.

On the basis of these results and previous experience in our laboratory (synthesis and molecular modelling of cantharidin inhibitors at PP1 and PP2A), we have designed a series of analogues which are more active and selective, whilst retaining the desirable properties of stability and cell permeability.

The synthetic pathways to these analogues are shown in schemes 1-3. Each scheme allows for modification of the basic skeleton, and in some cases the insertion of beneficial feature that were present in the more complex natural toxin(s) (eg okadaic acid, calyculin, microcystin, etc). These features will enhance selectivity and potency.

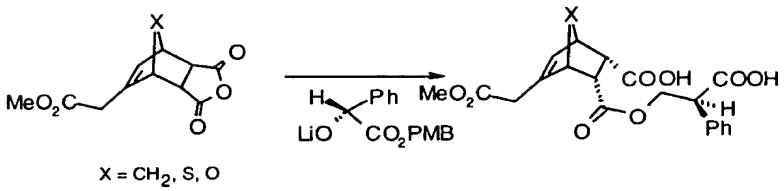


Example 3

Synthetic development of a series of PP1 and PP2A analogues of cantharidin.

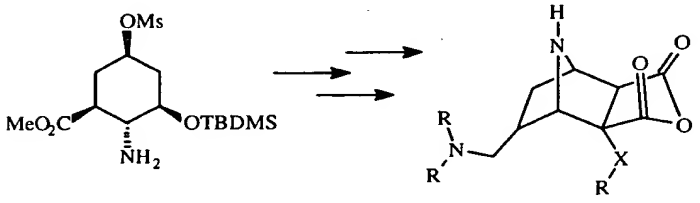
(i) Diels-Alder addition (maleic anhydride) and subsequent manipulations of X; (ii) Diels-Alder addition (substituted maleic anhydrides), introduction and manipulation of Z (Z = hydrophobic tail; eg long chain nitrile: cf Calyculin A, long chain terminating in a spiro acetal: cf Tautomycin, Okadaic acid; long chain terminating in an aromatic ring: cf Adda in Microcystin-LR; (iii) stereospecific ring opening of the anhydride allowing further manipulations of the newly released functional groups (see scheme 2).

In this instance we have developed synthetic protocols in our laboratory that allow the facile assembly of these analogues. Biological evaluation and molecular modelling of the most active molecules will allow our hypothesis to be evaluated.



Example 4

A specific example of one class of cantharidin analogue that shows promise as a selective inhibitor of protein phosphatases 1 and 2A.



Example 5

Stereospecific route towards 7-azabicyclo[2.2.1]heptanes

We have shown that the introduction of the bridgehead nitrogen improves the potency, selectivity and stability of similar analogues, the above pathway has been developed to further improve the bio-activity of these analogues. The synthetic routes alluded to herein have been developed and will allow the rapid assemble of the target molecules.

Those agents which meet the requirements of being stable, specific, potent, and membrane permeable protein phosphatase inhibitors are screened for their anti-cancer activity.

Example 6

Biochemistry

All synthesised compounds were tested for their ability to inhibit protein phosphatases 1 and 2A. Initial investigations were carried out at 100 mM. Promising analogues were then assayed in triplicate for estimation of IC_{50} values.

Protein phosphatase 1 and 2A were partially purified from chicken skeletal muscle essentially as described by Cohen Protein phosphatase activity was measured at 37°C in 50 mM Tris-HCl buffer (pH 7.4), 0.1 mM EDTA, 5 mM caffeine, 0.1% 2-mercaptoethanol and 1 mg/ml bovine serum albumin using 30 mg [^{32}P]-phosphorylase as substrate. The total assay volume was 30 ml. The assay conditions were restricted to 20% dephosphorylation to ensure linearity and inhibition of protein phosphatase activity was

determined by including cantharidin or its analogues at the required concentrations in the reaction buffer. Reactions were terminated by the addition of 0.1 ml ice cold 20% trichloroacetic acid. Precipitated protein was pelleted by centrifugation and the radioactivity in the supernatant measured by liquid scintillation counting. Data is expressed as the percentage inhibition with respect to a control (absence of a competing compound) incubation.

Example 7

Screening various PP1 and PP2A inhibitors for anti-cancer activity

(a) Cytotoxicity of protein phosphatase inhibition:

Those PP1 and PP2A inhibitors which fulfil the requirements detailed above were tested in various cancer cell lines. The cells lines chosen for study included both haematopoietic and solid tumour cell lines with varying p53 status and include:

L1210 (murine leukaemia, p53 wildtype),
HL60 (human leukaemia, p53 nul),
A2780 (human ovarian carcinoma, p53 wildtype)
ADDP (cisplatin resistant A2780 cells, p53 mutant),
SW480 (human colon carcinoma, p53 mutant)
WiDr (human colon carcinoma, p53 mutant)

Anticancer screening of the protein phosphatase inhibitors is assessed using the MTT assay. This assay determines cell viability by the ability of mitochondrial dehydrogenase to produce formazan crystals from 3-(4,5-

dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide. The viable cell number/well is directly proportional to the production of formazan, which following solubilization, can be measured spectrophotometrically (540nm). This technique is also used by the National Cancer Institute to screen for new anticancer agents.

(b) Abrogation of the G_2 checkpoint:

The ability of the protein phosphatase inhibitors to abrogate the G_2 checkpoint of the cell cycle may be determined by cell cycle analysis using flow cytometry. Briefly, asynchronous L1210 cell cultures are harvested 24h after 8Gy irradiation and/or 24h incubation with the protein phosphatase inhibitor. This cell line is p53 wildtype and we have shown that 24h after exposure of these cells to 8Gy of radiation induces cell cycle arrest in both the G_1 and G_2 phases of the cell cycle, as shown *below*.

If the protein phosphatase inhibitor abrogates the G_2 checkpoint then the L1210 cells will not arrest in the G_2 phase of the cell cycle and the cells will continue through the cell cycle and accumulate in the G_1 phase of the cell cycle only. Cell cycle analysis using propidium iodide labelling of DNA has been used extensively in our laboratory to assess the effect of specific anticancer agents that induce S-phase cell cycle arrest and apoptotic cell death (Sakoff, Ackland and Stewart, 1998). We have direct and unlimited access to radiation facilities at the Mater Hospital and limited access to a Becton Dickinson

FACScan and Cell Quest software.

(c) Combination studies:

The cell lines listed above are exposed continuously to cisplatin and the phosphatase inhibitor in various drug ratio combinations for 72h and then assayed for cytotoxicity. Similarly, the cells are exposed to 8 Gy of radiation and incubated with the phosphatase inhibitor and assessed for cytotoxicity at 72 h.

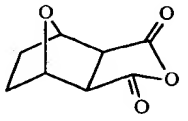
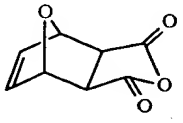
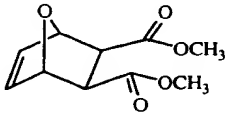
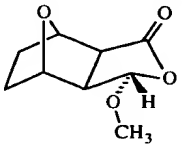
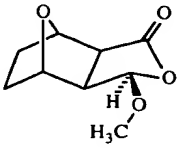
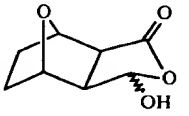
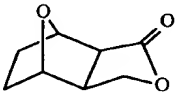
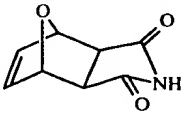
Example 8

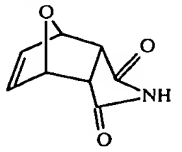
Results and Discussion

Anhydrides and simple analogues were synthesised according to literature procedures (Eggelte et. al), and then subjected to a PP1 and PP2A bio-assay (see biochemistry) to determine their ability to inhibit these enzymes. The results of initial screening at 100 mMs are shown in table 1, along with IC50 values in some instances.

Of the compounds listed in table 1, only **1**, **2** show any significant inhibition of PP2A, at 97% respectively (with little selectivity apparent for either enzyme). Interestingly the bioisoseteric replacement of the anhydride oxygen atom of **1** results in a complete loss of inhibition. Indeed no modification of the cyclic anhydride, is tolerated, and consequently results in no inhibition of PP2A.

Table 1. The inhibition of protein phosphatase 1 and 2A by anhydride modified cantharidin analogues.

Compound	Inhibition of PP1 (%)	Inhibition of PP2A (%)	Selectivity PP2A/PP1
 **1**	90 IC_{50} 2.4 μM	97 IC_{50} 2.1 μM	0.875
 **2**	ND	95	
 **3**	46 IC_{50} 50μM	6 IC_{50}>10,000μM	>200
 **4**	13	11	
 **5**	15	8	
 **6**	9	11	
 **7**	ND	21	
 **8**	ND	15	

 **9**	ND	4	

Previously we have shown that analog **2** undergoes a rapid conversion to the dicarboxylic acid under assay conditions. We thus examined the stability of the non-active analogues (in table 1) and found that they were stable under assay conditions showing no decomposition, in fact **5** can be synthesised via the Diels-Alder reaction in water.(Eggelte et. al).

Table 2. Effects of anhydride to dicarboxylic acid on the inhibition of PP2A.

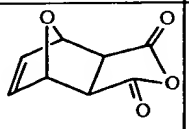
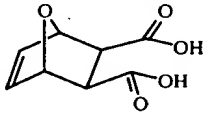
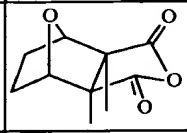
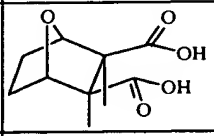
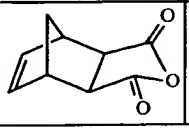
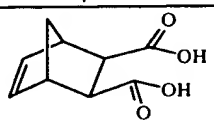
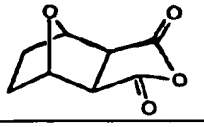
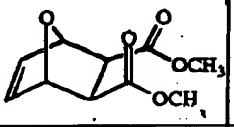
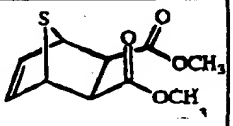
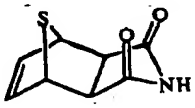
Entry	Anhydride	Inhibition (%)	Carboxylic acid	Inhibition (%)
1		97 (This work)	 **10**	80
2		92-95		92-95
3		48		17

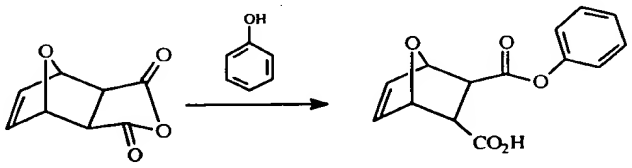
TABLE 3 Inhibition of PP1 and PP2A by selected cantharidin analogues.

Entry	Compound	Inhibition of PP1(%)	Inhibition of PP2A(%)	Selectivity PP2A/PP1
1		90 (IC_{50}2.4μM)	97 (IC_{50}2.1μM)	0.875
2		46 (IC_{50}50μM)	6 (IC_{50}>10000μM)	>200
4		3	3	Not determined
5		15	69	Not determined

In all instances, the corresponding dicarboxylic acid derivatives display lower inhibitory values at PP2A. Even though the anhydrides undergo a facile ring opening to the dicarboxylic acids, the original conformation presented at the active site must also play a role in determining the overall level of inhibition. Consequently, we believe that the conformation of anhydride carbonyl groups is more favourable for inhibition (essentially only one conformation presented at the active site), than that of the dicarboxylic acid (four possible minimum energy conformations, data not shown).

In an attempt to determine the feasibility anhydride opening via nucleophilic attack from Tyr272, we conducted

a series of model experiments in which **2** was allowed to stand in a chloroform solution of phenol. This mixture was examined periodically by ^{1}H NMR spectroscopy and showed the growth of a new species over a period of time (ca 10 days). Further analysis indicated the presence of a phenolate ester of norcantharidin (scheme 4). Consequently, a metal assisted or nucleophilic attack under physiological conditions represents a possible mode of assisted ring opening with the anhydride held in a favourable conformation within the active site. In turn the resultant diacid rapidly binds in a more favourable manner.



Scheme 4

The results presented herein indicate that cantharidin analogues, via anhydride opening are more potent inhibitors of PP2A. Analogues in which the anhydride moiety has been modified preventing a facile ring opening (except where otherwise indicated) are extremely poor inhibitors of PP2A.

However, the most interesting result reported herein (see table 1) is the selective inhibition of PP1 by the dimethyl ester (**3**). Simple diesterification of **2** has completely reversed the previously reported PP2A selectivity (ca 10 fold) of norcantharidin for PP2A to

yield, what we believe to be, the first selective small synthetic molecule for the inhibition of either PP1 or PP2A. Again this suggests that presentation of a diacid moiety to the active site is crucial for the inhibition of PP2A. No such restrictions are apparent with the limited structure activity data for PP1.

A synthetic inhibitor such as **3** represents a significant advance on the currently widespread inhibitors of PP1 and PP2A. We are currently conducting further studies utilising **3** as a lead compound to develop more potent inhibitors of protein phosphatase 1.

In conclusion, we have demonstrated that for inhibition at PP2A a facile ring opening of the anhydride moiety is crucial, with no structural modification tolerated. Also, that modification of the dicarboxylic acid moiety gives rise to a PP1 selective compound.

The above describes some embodiments of the present invention. Modifications obvious to those skilled in the art can be made without departing from the scope of this invention.

Industrial Applicability

It should be clear that the present invention will find light applicability, especially in the medical and veterinary fields.

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McCluskey, A; Taylor, C; Quinn, R J; Suganuma, M; Fujiki, H; *Bioorg. Med. Chem. Lett.*, **1996**, *6*, 1025.

Eggelte, T A; de Koning, H; Huisman, H O; *Tetrahedron*, **1973**, *29*, 2445; **3** see: Tamura, Y; Imanishi, K; Miki, Y; Ikeda, M; *Synthesis*, **1977**, 559; **5** & **9** see: Kwart, H; Burchuk, I; *J. Am. Chem. Soc.*, **1952**, *74*, 3094.

Goldberg, J; Huang, H-B; Kwon, Y-G; Greengard, P; Nairn, A C; Kuriayan, J; *Nature*, **1995**,*376*, 745.

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Sodeoka, M; Baba, Y; Kobayashi, S; Hirukawa, N; Bioorg. Med. Chem. Lett., 1997, 7, 1833.

Dated this 14th day of July 1998

THE UNIVERSITY OF NEWCASTLE RESEARCH ASSOCIATES LIMITED

By their Patent Attorneys

~~GRIFFITH HACK~~ BALDWIN SHELSTON WATERS

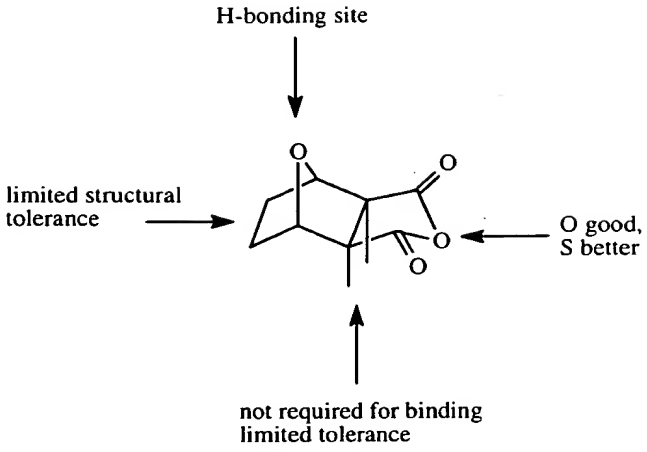

H-bonding site
limited structural tolerance
O good, S better
not required for binding limited tolerance

FIGURE 1

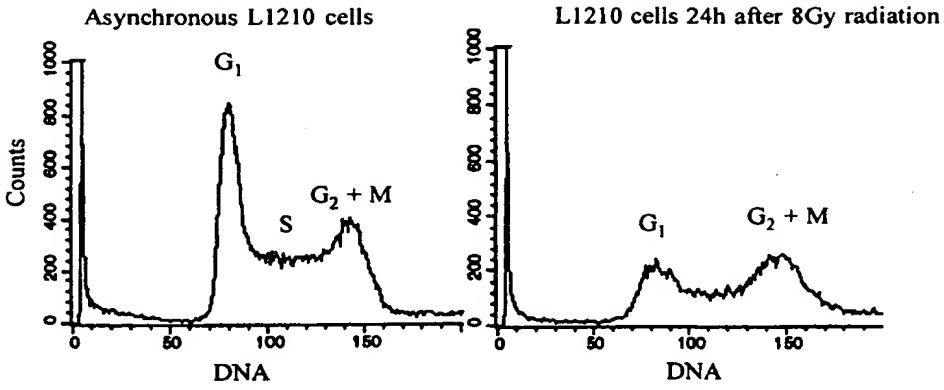
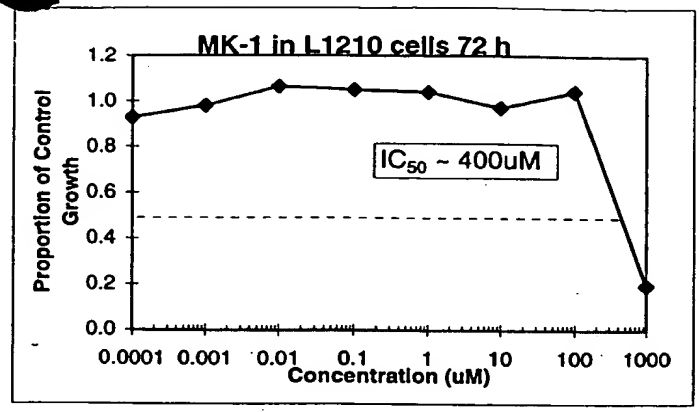
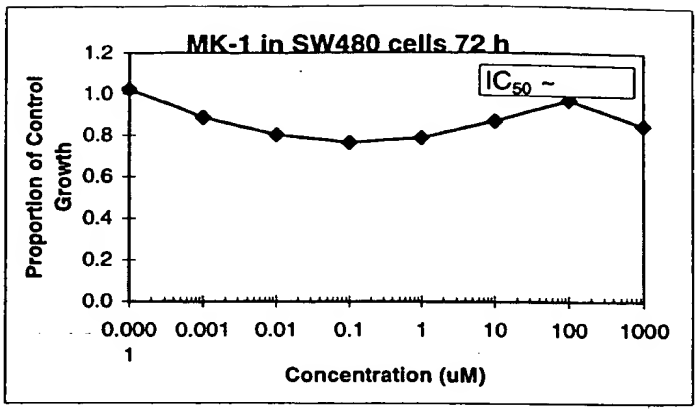
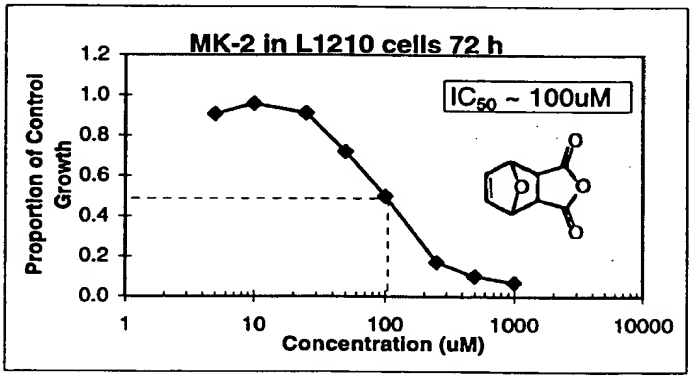
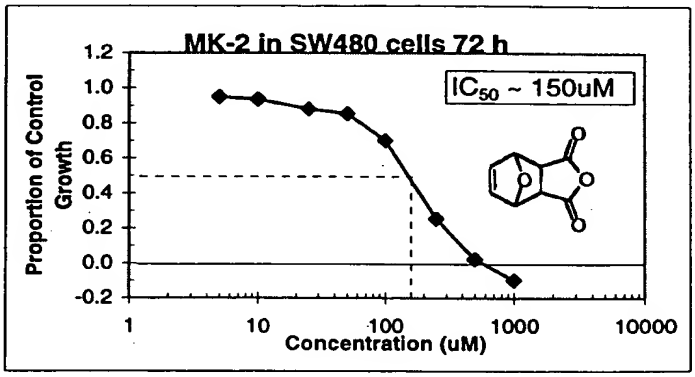

Asynchronous L1210 cells
L1210 cells 24h after 8Gy radiation
Counts
0
200
400
600
800
1000
G1
S
G2 + M
0
50
100
150
DNA
G1
G2 + M
0
50
100
150
DNA

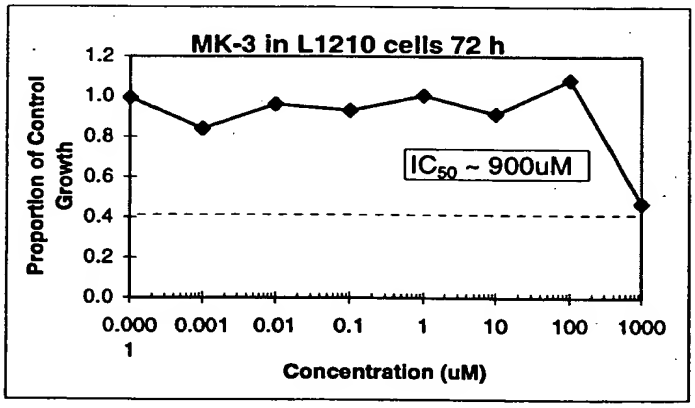
FIGURE 2

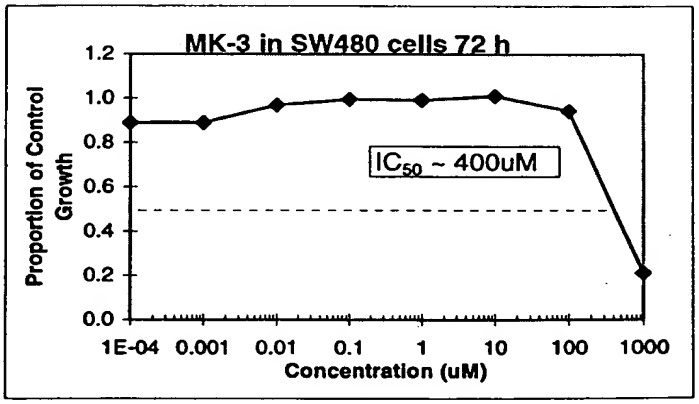

MK-1 in L1210 cells 72 h
Proportion of Control Growth
IC50 ~ 400uM
Concentration (uM)

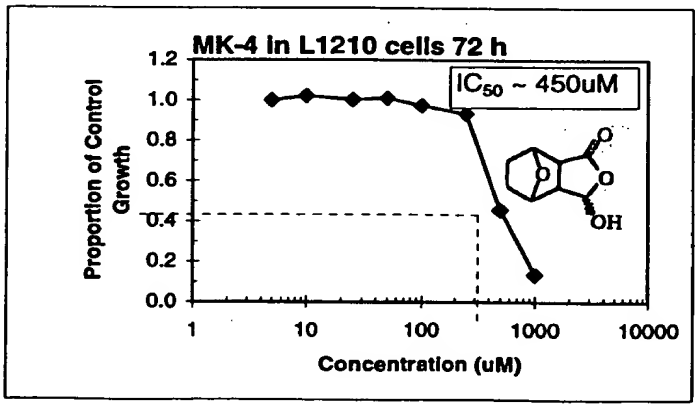

MK-1 in SW480 cells 72 h
Proportion of Control Growth
IC50 ~
Concentration (uM)


MK-2 in L1210 cells 72 h
Proportion of Control Growth
IC50 ~ 100uM
Concentration (uM)


MK-2 in SW480 cells 72 h
Proportion of Control Growth
IC50 ~ 150uM
Concentration (uM)


MK-3 in L1210 cells 72 h
Proportion of Control Growth
IC50 ~ 900uM
Concentration (uM)


MK-3 in SW480 cells 72 h
Proportion of Control Growth
IC50 ~ 400uM
Concentration (uM)


MK-4 in L1210 cells 72 h
Proportion of Control Growth
IC50 ~ 450uM
OH
Concentration (uM)

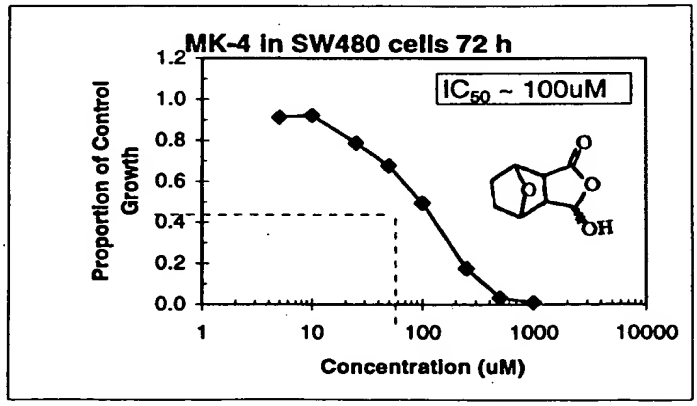
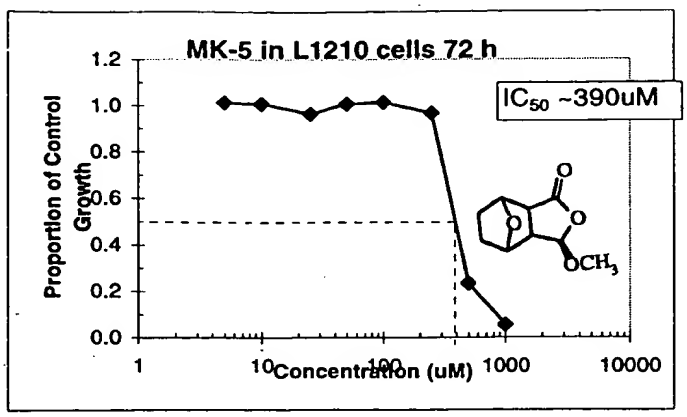
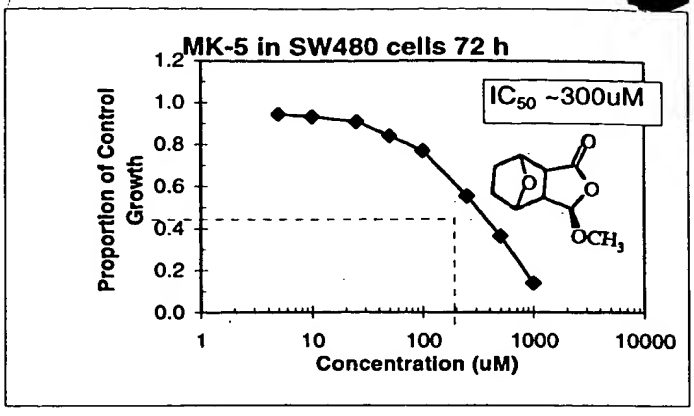
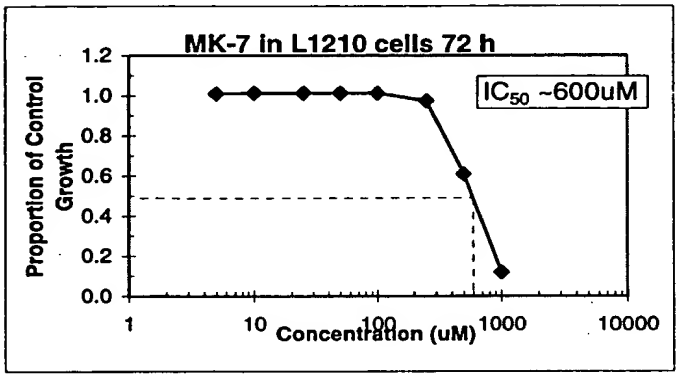
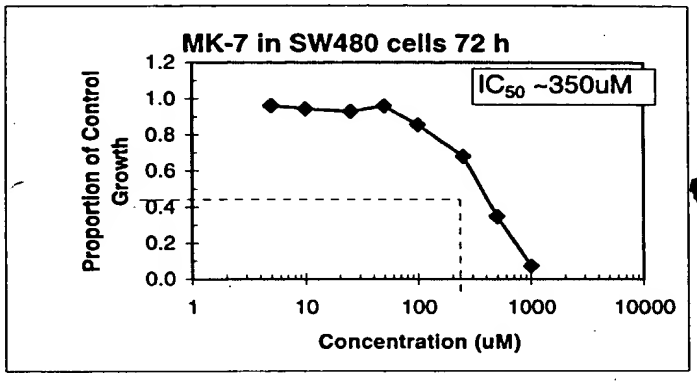

MK-4 in SW480 cells 72 h
Proportion of Control Growth
IC50 ~ 100uM
OH
Concentration (uM)

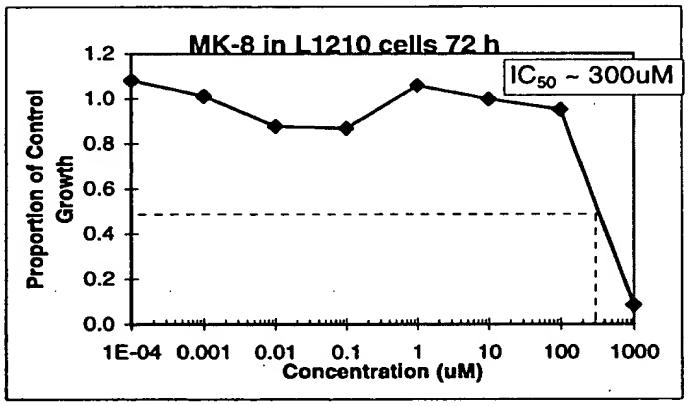
FIGURE 3A

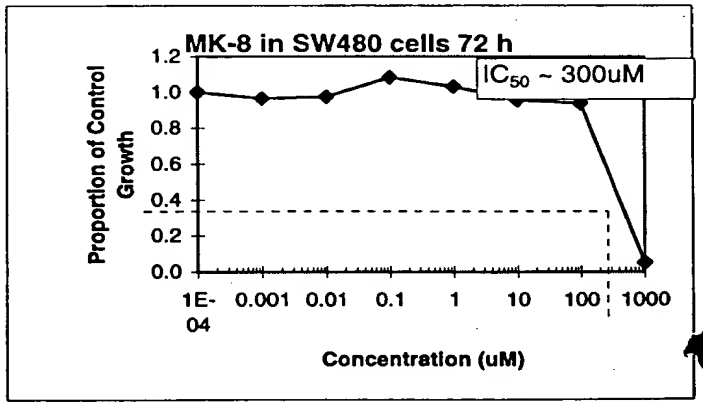

MK-5 in L1210 cells 72 h
IC50 ~390uM
Proportion of Control Growth
Concentration (uM)
OCH3

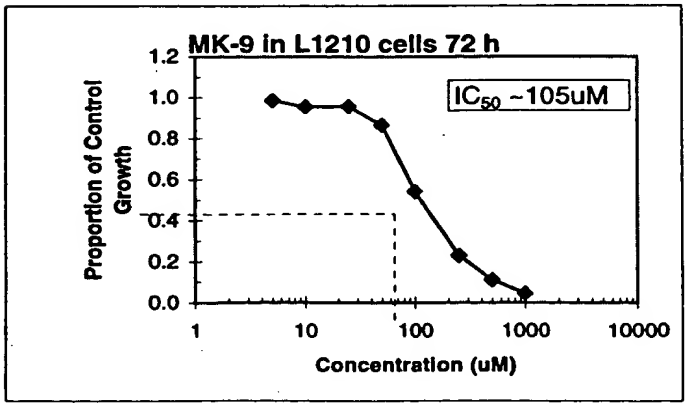

MK-5 in SW480 cells 72 h
IC50 ~300uM
Proportion of Control Growth
Concentration (uM)
OCH3


MK-7 in L1210 cells 72 h
IC50 ~600uM
Proportion of Control Growth
Concentration (uM)


MK-7 in SW480 cells 72 h
IC50 ~350uM
Proportion of Control Growth
Concentration (uM)


MK-8 in L1210 cells 72 h
IC50 ~ 300uM
Proportion of Control Growth
Concentration (uM)


MK-8 in SW480 cells 72 h
IC50 ~ 300uM
Proportion of Control Growth
Concentration (uM)


MK-9 in L1210 cells 72 h
IC50 ~105uM
Proportion of Control Growth
Concentration (uM)

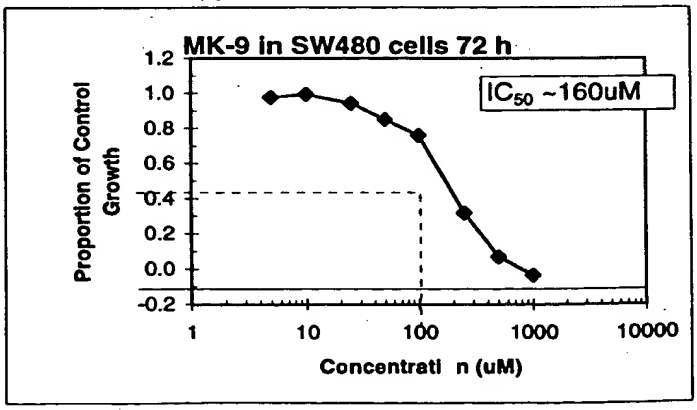

MK-9 in SW480 cells 72 h
IC50 ~160uM
Proportion of Control Growth
Concentrati n (uM)

FIGURE 3B